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Patterns of diversity, endemism and specialization in the root symbiont communities of alder species on the island of Corsica

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Summary

- We investigated whether the diversity, endemicity and specificity of alder symbionts could be changed by isolation in a Mediterranean glacial refugium. We studied both ectomycorrhizal (EM) fungi and nitrogen-fixing actinobacteria associated with alders, and compared their communities in Corsica and on the European continent.
- Nodules and root tips were sampled on the three alder species present in Corsica and continental France and Italy. Phylogenies based on internal transcribed spacer (ITS) and a multilocus sequence analysis approach were used to characterize fungal and *Frankia* species, respectively. Patterns of diversity, endemism and specialization were compared between hosts and regions for each symbiont community.
- In Corsica, communities were not generally richer than on the mainland. The species richness per site depended mainly on host identity: *Alnus glutinosa* and *Alnus cordata* hosted richer *Frankia* and EM communities, respectively. Half of the *Frankia* species were endemic to Corsica against only 4% of EM species.
- Corsica is not a hotspot of diversity for all alder symbionts but sustains an increased frequency of poor-dispersers such as hypogeous fungi. Generalist EM fungi and host-dependent profusely sporulating (Sp+) *Frankia* were abundantly associated with Corsican *A. cordata*, a pattern related to a more thermophilic and xerophylic climate and to the co-occurrence with other host trees.

Introduction

Alders are involved in a tripartite symbiosis with ectomycorrhizal (EM) fungi and nitrogen-fixing actinobacteria belonging to the genus *Frankia*. Compared to other trees, alders associate with few EM genera, some of them being strictly or mainly specific (Molina, 1981; Kennedy *et al.*, 2015). Similarly, alders associate with a specific clade of *Frankia* whose strains are shared with a limited number of other actinorhizal host plants (Normand *et al.*, 1996; Dawson, 2007). Among alders, the two subgenera *Alnus* and *Alnobetula* associate mainly with distinct EM (Rochet *et al.*, 2011; Roy *et al.*, 2013) and *Frankia* species (Pozzi *et al.*, 2015; Cotin-Galvan *et al.*, 2016). Few EM or *Frankia* species associate strictly with a single alder species and rather the host species influences the composition of its symbiont community (Lipus & Kennedy, 2011; Põlme *et al.*, 2013; Roy *et al.*, 2013). The reasons behind alder specificity have been intensely studied and tested, especially for EM fungi. Studies on European alders have demonstrated that the specificity pattern resulted from several events of host-tracking and host shifts (Rochet *et al.*, 2011). Other studies also demonstrated that alder phylogeny was strongly correlated with EM and *Frankia* community structures at a global scale (Põlme *et al.*, 2013, 2014). Apart from the host plant, other factors such as soil parameters, altitude and climate may also select adapted symbionts and explain some of alder's specificity. For instance, EM associated with alders are able to grow in high nitrate and low pH conditions, while other EM fungi show limited growth in these conditions (Huggins *et al.*, 2014). Soil type, acidity and moisture also shape part of *Frankia* diversity (Schwintzer, 1990; Markham & Chanway, 1998). Finally, co-migration or co-invasion events have probably maintained this specificity, as suggested for EM fungi in North America and New Zealand, and for *Frankia* globally (Kennedy *et al.*, 2011; Põlme *et al.*, 2014; Bogar *et al.*, 2015).

Several exceptions to the specificity pattern between alders and their symbionts have been identified in recent years. Alders can also associate with very generalist EM taxa that are often scarce but always present (Roy *et al.*, 2013), such as *Cenococcum geophilum* (Põlme *et al.*, 2013; Roy *et al.*, 2017). The study of co-occurring seedlings of *Alnus rhombifolia* and

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Betula occidentalis also revealed a few shared EM fungi, including *Alnicola* sp., and a significant effect of neighboring plants (Bogar & Kennedy, 2013). Similarly, the study of isolated *A. glutinosa* subsp. *barbata* populations in Georgia, a Tertiary and glacial refugium, revealed nonspecific species of *Inocybe* and *Tuber* spp. (Roy *et al.*, 2017). However, the detection of EM taxa shared with other host species remains rare, and is mainly limited to cases where alders are isolated or planted outside of their natural distribution range. These observations suggest that isolation or colonization of a new habitat may relax alder specificity and select new associations. Compared with EM fungi, no generalist *Frankia* has been described so far, as *Frankia* taxa associated with alders have never been detected on other host trees in the wild. The number of genera of actinorhizal plants able to associate with *Frankia* is also far lower and less diverse than genera of host plants associated with EM fungi. Thus, the co-occurrence of alders with another actinorhizal host is limited, although host-shifts of *Alnus*-infective *Frankia* have been described experimentally (Bosco *et al.*, 1992; Lumini *et al.*, 1996). As a consequence, isolated alder stands do not recruit generalist *Frankia* strains, as shown in Mexico and Georgia, where unique *Frankia* species have been detected (Higgins & Kennedy, 2012; Roy *et al.*, 2017). Moreover, *Frankia* sequences from Georgia were phylogenetically distant from other *Frankia* sampled worldwide. These observations suggest that refugia, or at least isolated populations, might host endemic *Frankia* species and show a strong biogeographic pattern of distribution, as shown for bacteria specialized toward a specific niche (Angel *et al.*, 2010; Brown *et al.*, 2012). Conversely, EM fungal species have a broader distribution, with only a few exceptions reported for Corsica (Rochet *et al.*, 2011).

The biogeographic pattern of symbiotic fungi and bacteria may also depend on their biology. Indeed, the ability to migrate with or without the host is impacted by life-history traits, particularly dispersal and host-dependency. Sporulation is a major component of microbe dispersal (Huang & Hull, 2017). Both EM fungi and *Frankia* are able to sporulate, yet with major differences. Among EM fungi, hypogeous taxa such as *Alpova* or *Melanogaster* sp. can be dispersed by small mammals and are less likely to migrate than wind-dispersed epigeous fungi. Because actinorhizal nodules closely resemble hypogeous structures, *Frankia* would also have more limited dispersal abilities as compared to epigeous fungi. Among *Frankia,* dispersal may also depend on contrasting sporulation traits. Indeed, some strains sporulate profusely within nodular cells (Sp+), while other strains do not (Sp−). Host-dependency also differs: EM fungi and Sp+ *Frankia* are considered to be obligate symbionts, while Sp− *Frankia* strains can be cultured and live as saprobes in soil (Schwintzer, 1990; Pozzi *et al.*, 2015; Cotin-Galvan *et al.*, 2016). These life history traits could lead to distinct symbiont distributions and impact alder symbiont communities, particularly where alders have been isolated, such as in glacial refugia or on islands.

In Europe, pollen records and phylogeographic studies have revealed several southern glacial refugia for alders, such as the Iberian peninsula, the Apennine mountains, Corsica (an island), North Africa, and the Balkan and Anatolian Peninsulas (King & Ferris, 1998; Douda *et al.*, 2014; Havrdová *et al.*, 2015; Mandák *et al.*, 2016), together with a few northern refugia (Douda *et al.*, 2014; Mandák *et al.*, 2016). Corsica was isolated from the continent (*c.* 80 km) 20–23 Myr ago (Ma) (Blondel & Aronson, 1999) and is known as a hotspot of plant diversity and endemism (Medail & Quezel, 1997), where boreo-arctic plants took refuge from the Tertiary glaciations and now co-occur with Mediterranean and temperate elements (Jeanmonod & Gamisans, 2007). Three alder species are present in Corsica and show distinct degrees of isolation from the mainland. *Alnus cordata* (Loisel.) Duby is native both to Corsica and southern Italy, where it grows from 150 to 2000 m (Gamisans, 1999). *Alnus glutinosa* (L.) Gaertn is a widespread Eurasian species and has colonized Corsican riparian forests, marshes and peat bogs from sea level up to 1300 m (Gamisans, 1999). *Alnus glutinosa* and *A. cordata* can co-occur and their distribution ranges partly overlap. Finally, the subalpine shrub *Alnus alnobetula* subsp. *suaveolens* (Req.) Lambinon & Kerguélen is endemic to Corsica, and diverged from the continental alpine subspecies *A. alnobetula* subsp. *alnobetula* (Ehrh.) K. Koch *c.* 1.1 Ma (Rochet *et al.*, 2011). These features invite study of alder symbiont communities in Corsica, and we investigated if they are particularly diverse and possibly endemic, and if host shifts could have occurred between the three alder species. No reports were available for *Frankia*. Previous studies on EM fungi of alder that partly investigated Corsican populations revealed only two endemic EM species, *Alpova corsicus* and *Melanogaster rivularis*, and highlighted the heterogeneity of EM communities and the abundance of generalist taxa such as ascomycetes on *A. cordata* roots (Moreau *et al.*, 2011; Roy *et al.*, 2013). In Corsica, *A. cordata* forms very typical mixed woods with *Castanea sativa* at mid-altitudes (Gamisans, 1999), while it forms rather monodominant stands along the Italian coast (Ducci & Tani, 2009). While mixed woods may affect the symbiont communities of alders in Corsica, no particular link was detected between *C. sativa* and *A. cordata* EM communities, probably due to the limited number of sites investigated (Taudiere *et al.*, 2015). This study also suggested the possible sharing of EM fungi between the three alder hosts. However, this situation was not compared with the mainland.

The aim of this study was to investigate the diversity, endemism and specialization patterns of alder symbionts in Corsica. We sampled both *Frankia* and EM fungi on the three alder species and compared Corsican communities with communities sampled from the continent, in France and Italy. To discuss species distributions for the two symbionts, we chose the same evolutionary species concept and used the most appropriate, yet different, molecular markers to delineate species for each symbiont. We hypothesized that alder symbiont communities in Corsica might be more species-rich and include more endemics, and that co-occurring alder or other host tree species may favor symbiont sharing. These questions will be discussed in the context of host history and current distribution, and of symbiont life history traits.

Materials and Methods

Sampled sites

Seventy sampling sites were investigated on Corsica and in continental France and Italy, from lowland to subalpine habitats (Fig. 1; Supporting Information Table S1). Sampling was carried out in November 2011 in Corsica and in May 2012 in Italy. Data from sites previously studied were included as the sampling protocol was identical (Roy *et al.*, 2013; Pozzi *et al.*, 2015). At each site, one or two species of alder were present, either monodominant or scattered in mixed forests. Relevant climatic data were retrieved from the Worldclim database (http://worldclim.org) using the RASTER package in R (Hijmans & van Etten, 2012).

Symbiont and soil sampling

For each site, four to six trees – separated from each other by at least 10 m – were sampled from two opposing sampling points. Approximately 50 cm of roots harboring both EM root tips and nodules were collected per tree. For the most recent sampling sites (Table S2) a soil core of 3 cm diameter and 10 cm depth was driven into the soil close to the tree roots. Roots, nodules and soils were kept at 4°C until processing in the lab. Soils from the same sampling site were pooled, sifted at 2 mm and dried at 60°C before physiochemical analysis. Soil analyses were provided by ECOLAB (Toulouse, France) or retrieved from previous studies (Rochet *et al.*, 2011; Roy *et al.*, 2013). Approximately 16 EM root tips and four nodule lobes per tree were preserved, as previously described (Roy *et al.*, 2013; Pozzi *et al.*, 2015).

Molecular biology and sequencing of symbionts

For fungi, DNA extraction was performed on each EM root tip using Promega's Wizard genomic DNA purification kit, following the manufacturer's instructions. The fungal internal transcribed spacer (ITS) region was amplified as in Rochet *et al.* (2011). Putative new symbionts for alders were confirmed by sequencing the plant ITS to check the host identity. Sequencing of the amplification product was done by MilleGen (Labège, France). Sequences were checked and cleaned using GENEIOUS 6.1.8 (Kearse *et al.*, 2012). All new fungal sequences produced in this study were deposited with GenBank under accession numbers KU924024–KU925159 and KY131803 (Table S3). For *Frankia*, DNA extraction was performed on each lobe individually, as previously described (Pozzi *et al.*, 2015). For the three housekeeping genes, *pgk*, *dna*A and *fts*Z, portions were amplified and sequenced with specific primers following Pozzi *et al.* (2015). Sequencing of the amplification product was done by Biofidal-DTAMB (Villeurbanne, France). Sequence quality was verified using 4PEAKS v.1.7.2 (Griekspoor & Groothuis, 2007). All *Frankia* sequences were deposited with EMBL under accession numbers LT555594–LT555681 (*dnaA*), LT555682–LT555771 (*ftsZ*) and LT555772–LT555865 (*pgk*).

Identification of symbionts

We chose the same evolutionary species concept for both symbionts where species are sequence-based monophyletic clades of strains sharing a common ancestor. The molecular markers used to call operational taxonomic units (OTUs) were chosen based on their accuracy, reliability and appropriateness for each symbiont and were thus different. Yet, all the OTUs were delineated at the same taxonomic rank – namely species – enabling a valid comparison between EM and *Frankia* species. Sequences were assigned to a Linnaean name after phylogenetic analyses. They were designated by an arbitrary numbering system when several unnamed species were characterized in the same genus, particularly within the genus *Frankia* for which only a small portion of the existing genomospecies has been named.

For fungi, using BLAST on GenBank and UNITE, it was possible to assign sequences to putative genera and to UNITE species hypotheses (Table S3). The closest sequences (97–100% identical) and some more distant sequences (with a reliable Linnaean name) were used for the alignment. *Alnicola badiofusca* and *A. umbrina*, two species supported by morphology and ecology but not differentiated by ITS (Moreau *et al.*, 2013), were treated as a species complex and discarded from the analyses. Alignments were made using MAFFT (Katoh & Standley, 2013). Phylogenies of each EM genus were constructed using maximum likelihood analysis under a GTR+G model of evolution in RAxML (Stamatakis *et al.*, 2008). The robustness of phylogenies was tested by rapid bootstrapping (1000 pseudoreplicates) on the CIPRES Science Gateway (Miller *et al.*, 2010). We compared the ITS sequences with data obtained from sporocarps and the phylogenies developed for several genera associated with alders (Rochet *et al.*, 2011), as recommended to delineate accurate fungal species (Taylor *et al.*, 2000) corresponding to unified species (De Queiroz, 2007). For *Tomentella* spp., sequences associated with a Linnean name were often missing, and OTUs were delineated as monophyletic clusters of sequences, supported by a > 80% bootstrap value and sharing ITS sequences of > 97% similarity.

For *Frankia*, we used multilocus sequence analysis (MLSA) widely recommended to delineate prokaryotic species (Gevers *et al.*, 2005). Housekeeping genes were chosen as, for *Frankia*, they better correlate with phylogenetic and whole-genome data (Sen *et al.*, 2014; A. C. Pozzi *et al.,* unpublished). Amino acid sequences of the three loci were aligned separately with MUSCLE v.3.8.31 (Edgar, 2004) and concatenated as nucleotide sequences in SEAVIEW v.4.4.2 (Gouy *et al.*, 2010) for a subset of 94 nodules including 15 reference strains. The Bayesian inference of phylogeny was computed as in Pozzi *et al.* (2015) on the CIPRES Science Gateway (Miller *et al.*, 2010). OTU delineation was based on an enlarged dataset (one to three loci for 224 root nodules, Table S4) and a similar phylogenetic analysis, enabling a comparison of new sequences with lineages described worldwide (Pozzi *et al.*, 2015). We computed a histogram of pairwise phylogenetic distances (Fig. S1) and determined the threshold of 0.05 substitutions per site as the best barcode gap value (Kekkonen & Hebert, 2014) to delimit OTUs corresponding to unified species

(De Queiroz, 2007). An OTU cladogram was also constructed from the phylogenetic distances.

Detection of spatial autocorrelation and geographic structure

To test whether symbiotic communities were spatially structured, a multivariate spatial autocorrelation test was performed (SPDEP package; Smouse & Peakall, 1999). Isolation by distance was tested through correlation between geographic distances (calculated from GPS coordinates; see Fig. 1 and Table S1) and Bray–Curtis distances through a Mantel test (Mantel, 1967).

Host and regional effects on alpha, beta and gamma diversities

For all diversity indices, we tested the influence of (1) host identity, with two factors: host subgenus and host species nested in subgenus, and (2) region, with two factors: Continent vs Corsica as the first regional level, and the difference between France and Italy nested in Continent as a second regional level. To compare the gamma (total) diversity between host and regions, species accumulation curves were computed. To estimate alpha diversity, species richness (number of species per site) and Shannon diversity indices were computed (diversity function of the VEGAN package in R; Dixon, 2003). The species richness was square-root-

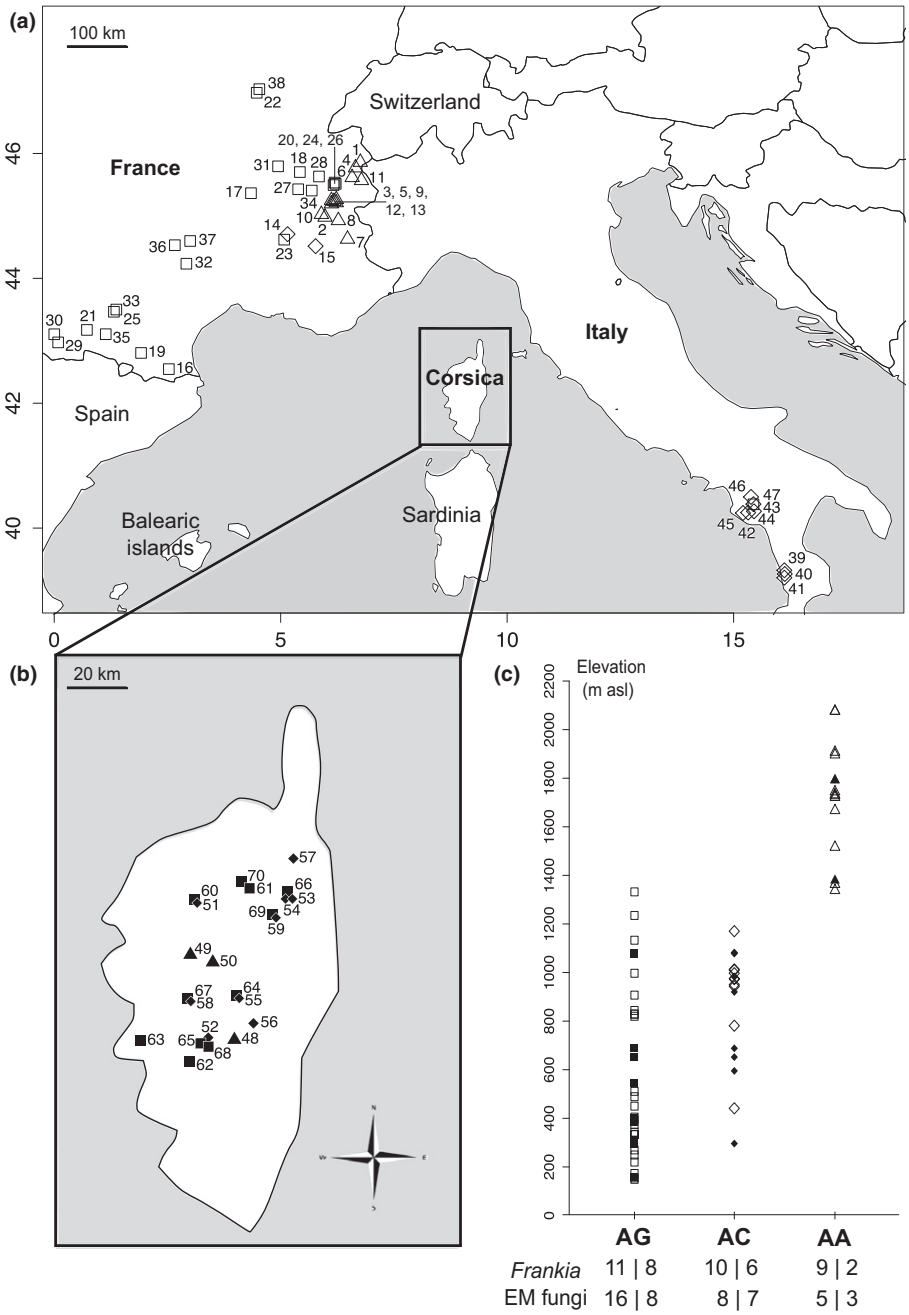

Fig. 1 Map of sampled populations. (a) Continental alder populations sampled in France and Italy. (b) Alder populations sampled in Corsica. (c) Elevation of sampled population (in metres above sea level (m asl)) for the three sampled alder species and number of sampled populations per symbiont (Continent|Corsica). (a–c) Open symbols, Continental populations; closed symbols, Corsican populations. Alder species: *Alnus glutinosa* (AG, squares); *A. cordata* (AC, diamonds); *A. alnobetula* (AA, triangles). EM fungi, ectomycorrhizal fungi. Population numbers refer to Supporting Information Tables S1 and S2.

transformed to meet the assumption of homoscedasticity. An analysis of variance (ANOVA) was used to test the effect of all factors on alpha diversity indices (Table 1). To estimate beta diversity (dissimilarity between communities), Bray–Curtis distances were calculated per site (Bray & Curtis, 1957). Variance analysis of beta diversity was performed through a multivariate permutation test (Adonis, VEGAN package in R; Table 2). A subsequent test was performed to check if beta diversity variance was homogeneous between hosts or regions (betadisper test followed by an ANOVA). The effect of host species and regional factors on site ordination was illustrated using nonmetric multidimensional scaling (NMDS, meta-MDS function, VEGAN package). The correlation between NMDS, host and regional factors as well as geographic and climatic variables was tested through a permutation analysis (envfit function, VEGAN package in R). Only significant factors or variables ($P > 0.05$) were represented.

Species distribution and detection of symbiont endemism and specificity

To illustrate the distribution of shared symbiont species between regions and hosts in our dataset, and detect putative endemic or specific species, the abundance of *Frankia* and EM fungi was calculated per region (Corsica vs Continent) and per host species to build a Venn diagram and hierarchical clustering, ranked by Bray–Curtis distances (Bray & Curtis, 1957).

Phylogenetic approaches and taxonomic knowledge helped to detect true endemics and determine the specificity level of each symbiont species. Classes of specialization were established as follows: (I) species-specialist: 100% of the species records found with a single *Alnus* species (*A. alnobetula*, *A. cordata* or *A. glutinosa*); (II) subgenus-specialist: 100% of the records found within the subgenus *Alnus* (i.e. *A. cordata* and *A. glutinosa* in our dataset, and mainly *A. incana* (L.) Moench in additional data); (III) alder-specialist: 100% of the records found with *Alnus* spp. with the exclusion of other trees and environmental samples without alders; (IV) generalist: at least one reliable record of species not related to alders; and (V) undetermined. Classes I, II and III were common to EM fungi and *Frankia*, the others only to fungi. To compare the respective specificity patterns of EM fungi and *Frankia*, the species abundance matrix was recoded with the five classes of specialization for a restricted number of sites where both symbionts were sampled (Table S5). Site ordination (NMDS) and correlation between NMDS, host and regional factors as well as geographic and climatic variables were tested as described above. Four soil variables were also tested (KCl pH, %N, %C and C : N ratio).

Distribution of short-distance dispersal symbionts

The effects of short-distance dispersal of Sp+ *Frankia* and hypogeous fungi were tested between Corsica and the Continent. For *Frankia*, the *in planta* sporulation phenotype was determined microscopically on stained nodule sections (Pozzi *et al.*, 2015). A nodule was considered Sp+ when at least one sporangium was observed out of 50 infected plant cells (Schwintzer, 1990). The proportion of Sp+ nodules was calculated per host and per

Table 1 ANOVA on alpha-diversity indices

Symbiont	Diversity index		df	Sum of squares	*F*-statistics	*P*-value	Significance
EM	Species richness (square root)	Host subgenus	1	0.148	0.297	0.589109	ns
		Host species	1	0.523	1.046	0.312620	ns
		Region, level 1	1	1.747	3.496	0.069050	ns
		Region, level 2	1	6.448	12.904	0.000907	***
		Residuals	39	19.488			
	Shannon	Host subgenus	1	0.0331	0.656	0.4230	ns
		Host species	1	0.0386	0.764	0.3875	ns
		Region, level 1	1	0.1717	3.401	0.0728	.
		Region, level 2	1	1.3532	26.806	7.14e-6	***
		Residuals	39	1.9688			
Frankia	Species richness (square root)	Host subgenus	1	0.488	2.956	0.0937	.
		Host species	1	0.086	0.519	0.4757	ns
		Region, level 1	1	0.178	1.080	0.3053	ns
		Region, level 2	1	0.641	3.881	0.0562	.
		Residuals	38	6.275			
	Shannon	Host subgenus	1	0.653	2.939	0.0946	.
		Host species	1	0.000	0.000	0.99446	ns
		Region, level 1	1	0.095	0.429	0.5167	ns
		Region, level 2	1	1.255	5.652	0.0226	*
		Residuals	38	8.436			

Analysis of variance (ANOVA) was performed on the extrapolated species richness and Shannon index of each symbiont species pool. Symbiont (species|populations): ectomycorrhiza (EM) alone (110|44), *Frankia* alone (22|43). For EM and *Frankia,* two (nos. 43 and 59) and four (nos. 14, 15, 24 and 47) outlier populations were removed, respectively. df, degrees of freedom. Bioregion factor is nested: region level 2 (Corsica, France, Italy) into region level 1 (island, continental). Host factor is nested: host species (*Alnus alnobetula*, *A. cordata*, *A. glutinosa*) into host subgenus (*Alnobetula*, *Alnus*). Significance: ns, not significant; ., $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 2 Multivariate permutation test on ectomycorrhizal (EM) and *Frankia* community dissimilarities (beta-diversity)

Symbiont	Factor tested	df	Sum of squares	*F*-statistic	R^2	*P*-value	Signifiance
EM	Host subgenus	1	1.1119	3.4300	0.07055	0.001	***
	Host species	1	1.1409	3.5195	0.07239	0.001	***
	Region, level 1	1	0.3573	1.1024	0.02267	0.331	ns
	Region, level 2	1	0.5083	1.5682	0.03225	0.053	.
	Residuals	39	12.6423		0.80214		
Frankia	Host subgenus	1	3.3736	13.5854	0.21078	0.001	***
	Host species	1	1.8202	7.3301	0.11373	0.001	***
	Region, level 1	1	0.6829	2.7499	0.04267	0.016	*
	Region, level 2	1	0.6920	2.7867	0.04324	0.011	*
	Residuals	38	9.4362		0.58958		

Multivariate permutation test was performed on dissimilarities of Bray–Curtis distance matrices for each of the symbiont communities. Symbiont (species|populations): ectomycorrhiza alone (110|44), *Frankia* alone (22|43). Symbiont: for EM and *Frankia,* two (nos. 43 and 59) and four (nos. 14, 15, 24 and 47) outlier populations were removed, respectively. df, degrees of freedom. Host factor is nested: host species (*Alnus alnobetula, A. cordata, A. glutinosa*) into host subgenus (*Alnobetula, Alnus*). Region factor is nested: region level 2 (Corsica, France, Italy) into region level 1 (island, continental). *P*-values assess the overall significance of all terms together. Significance: ns, not significant; ., $P<0.1$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

region. The equality of proportions was tested using a two-sample test with continuity correction (t.test function, STAT package in R). We also tested the correlation between the proportion of Sp+ nodules and *Frankia* alpha diversity by a linear model and a Spearman rank-order correlation test (cor.test function, STAT package in R). For EM fungi, we estimated the frequency of sequences identified as hypogeous fungi. The effect of host and region on their frequency at each site was tested by an ANOVA and completed with a Mann–Whitney test for pairwise differences, both computed in R.

Results

Molecular biology and sequencing of symbionts

For this study, 1139 EM root tips were collected in Corsica and 769 in Italy, of which 595 and 115 produced an EM sequence, respectively. In Italy, 45% of root tips were colonized by several fungi or were difficult to amplify and 33% of amplicons produced poor quality sequences. Sequences attributed to nonalder roots, that is 30 sequences belonging to Helotiales, Amanitaceae, Sebacinaceae, Boletaceae, Cantharellaceae and a Pezizales, as well as sequences attributed to alders but considered as endophytic rather than EM (Tedersoo *et al.*, 2009), that is 17 Helotiales, were discarded from the dataset. Our final dataset included 1343 EM and 224 *Frankia* sequences (Tables S3, S4).

Identification of EM symbionts

For fungi, 110 species were recognized (mean 99.1% similarity for the best BLAST, Table S3). The genera *Tomentella* and *Alnicola* were the most diverse (23 and 12 species, respectively), and the most abundant (351 and 186 sequences, respectively). Thirty-four species were not yet recorded on alders and half of them could not be named with certainty at the species level. In the genus *Lactarius*, the species identified as *L. lepidotus* (a specialist of *A. alnobetula* subsp. *alnobetula*) needed re-evaluation, as all sequences were clustered in two well-supported lineages (arbitrarily named '*L. lepidotus* 1' and '*L. lepidotus* 2'), both distinct from the two sister species *L. lilacinus* and *L. cuspidatoaurantiacus* (Fig. 2).

Identification of *Frankia* symbionts

For *Frankia*, a single concatenation of up to 2040 nucleotide positions was aligned and used to infer the phylogeny (Fig. 3b). The phylogenetic tree topology was highly supported by posterior probabilities. All Corsican symbionts belonged to the monophyletic lineage of *Alnus*-infective *Frankia* and grouped into five divergent clades (1, 5–8), of which three clades (6, 7 and 8) have not been described before. Clade 6 and subclade 8b were strictly Corsican. Strains associated with Corsican *A. alnobetula* subsp. *suaveolens* clustered in a monophyletic lineage (1a-AAS) close to two sister clades associated with the other subspecies, either *alnobetula* in Europe (1a-AAA) or *fruticosa* and *crispa* in North America (1a-AAF/AAC). Strains isolated from *A. cordata* were often shared between Corsica and Italy, except subclade 8b. For *A. glutinosa*, only clade 6 was endemic to Corsica. Twenty-four species were delineated among 224 sequences, 11 were singletons and three – 03, 20 and 23 – grouped together in *c.* 2/3 of the sequences (Fig. 3a). All the OTUs had species rank, as deduced from the reference strain positions in the phylogeny (Fig. 3b). As alder-infective *Frankia* species are mostly unnamed, the same is true for their phylogenetic clades and OTUs.

Autocorrelation test between sites and geographical structure

For EM fungi, the autocorrelation test was not significant ($P=8e\text{-}04$) and the spatial weight was not included in further analyses. For 70 out of 73 species (37 singletons were removed), the distribution was not spatially aggregated and the Geary–

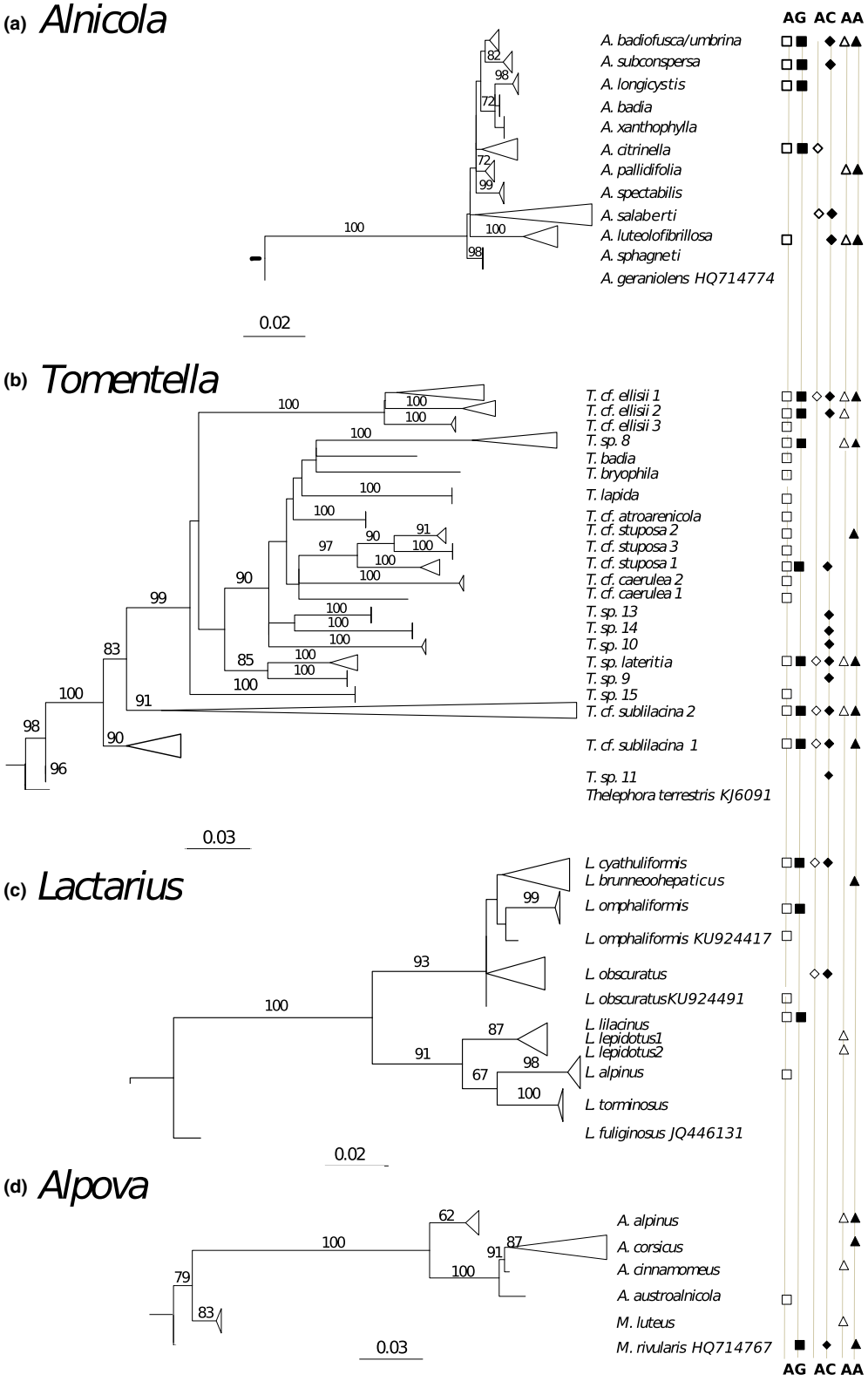

Fig. 2 Ectomycorrhizal (EM) fungi phylogenies. Phylogenies of EM genera associated with alders, based on the fungal internal transcribed spacer (ITS) regions and estimated using maximum likelihood: (a) genus *Alnicola*, (b) genus *Lactarius*, (c) genus *Alpova*, (d) genus *Tomentella*. (a–d) Strain origin (host plant): *Alnus glutinosa* nodules (AG, squares), *A. alnobetula* nodules (AA, triangles), *A. cordata* (AC, diamonds). Strain origin (region): continental France and Italy (open symbols) or Corsica (closed symbols). Node reliability was assessed using rapid bootstrapping (1000 pseudoreplicates). The scale represents the average number of substitutions per nucleotide.

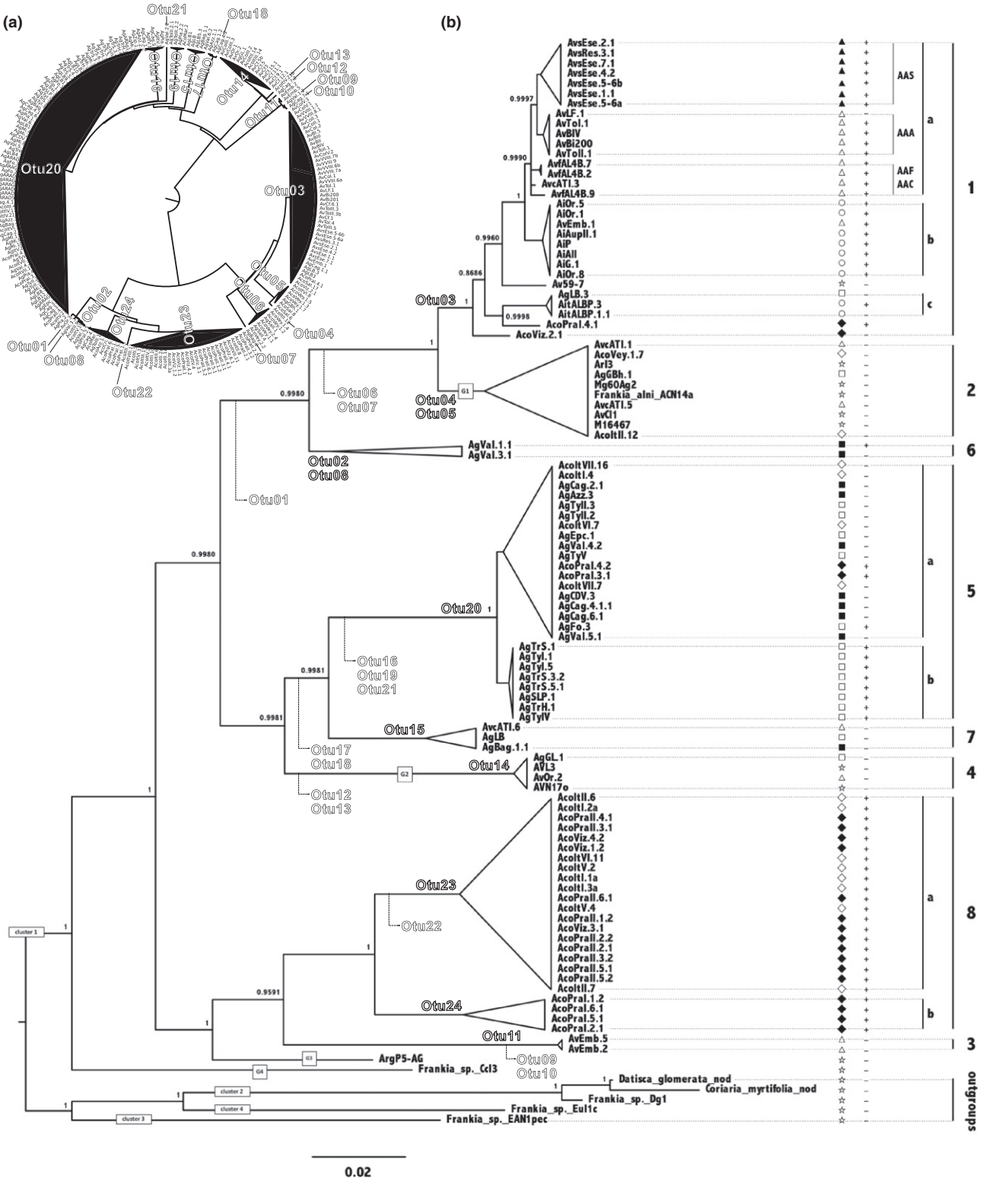

Fig. 3 *Frankia* operational taxonomic unit (OTU) cladogram and multilocus sequence analysis (MLSA) phylogeny. (a) Cladogram of *Frankia* molecular taxonomic units (OTUs) calculated from the hierarchical clustering of *dnaA*, *ftsZ* and/or *pgk* marker alignment distances at the 0.05 threshold. Branch lengths are proportional to alignment distances and the angle sector of an OTU is proportional to its abundance. OTUs reported outside of the circle were singletons. (b) MLSA phylogeny of *Alnus*-infective *Frankia* based on concatenated sequences of *dnaA, ftsZ and pgk* markers and estimated by Bayesian inference. Strain origin (host plant): *Alnus glutinosa* nodules (squares), *A. incana* nodules (circles), *A. alnobetula* nodules (triangles), *A. cordata* (diamonds), reference strains (stars). Strain origin (region): continental France and Italy (open symbols) or Corsica (closed symbols). Phenotype of *in-planta* sporulation: spore-positive (+), spore-negative (−). Clades 6–8 are defined in the present study; clades 1–5 were defined in a previous study (Pozzi *et al.*, 2015). G1–G4, genomospecies of *Frankia* cluster 1 (Fernandez *et al.*, 1989). Clusters 1–4, as defined in Normand *et al.* (1996). Node reliability was assessed using posterior probabilities. The scale represents the average number of substitutions per nucleotide.

Moran test was only significant ($P < 0.05$) for three species, *Alnicola salabertii*, *Lactarius obscuratus* and *Russula* sp. 2. No correlation was detected between geographic distance and beta diversity for EM (Mantel test, $r = 0.06$, $P = 0.12$). The *Frankia* dataset was not spatially autocorrelated, based on a multivariate randomization test ($P = 0.0905$). At the species level, only OTUs 3, 4 and 23 showed a spatially aggregated distribution (Geary–Moran test, $P < 0.05$). However, beta diversity and geographic distance were correlated (Mantel test, $r = 0.11$, $P = 0.02$).

Host and regional effects on gamma, alpha and beta diversities

Accumulation curves of *Frankia* reached a saturation plateau, while EM fungi did not (Fig. S2a). In Corsica, more EM species were associated with *A. cordata* compared to continental communities (Fig. S2b), the opposite pattern being observed for EM communities associated with *A. glutinosa*, while *A. alnobetula* subsp. *suaveolens* and subsp. *alnobetula* species accumulation curves did not differ and were instead influenced by sampling size. Overall, we did not detect a significant difference between Corsica and the Continent, nor a significant host effect on EM species richness and alpha diversity (Shannon index, Table 1). At the site level, EM species richness was not significantly higher in Corsica, and only Italian communities were less diverse and less species-rich. For *Frankia*, accumulation curves per host and per region followed a different trend compared to EM fungi. More species were associated with *A. glutinosa* in Corsica compared to the Continent and the opposite pattern was observed for *A. cordata* (Fig. S2c). Compared to these two host species, *A. alnobetula* subsp. *alnobetula* hosted fewer species than other alders on the Continent, and too few sites were investigated for *A. alnobetula* subsp. *suaveolens* to compare its accumulation curve. At the site level, *Frankia* communities hosted significantly more species in France compared to Italy (Table 1). The Shannon diversity index followed the same pattern as EM fungi, and only Italian communities were less diverse.

Beta diversity was not significantly different between EM communities in Corsica and the Continent, but slightly distinct when comparing Corsica, Italy and continental France (Tables 2, S6). Differences between communities were partly explained by the host subgenus (7%) and host species (7%) factors, yet 80% of the variation remained unexplained (Table 2). Communities associated with *A. cordata* were particularly distinct between Corsica and Italy (Fig. 4a). For *Frankia*, 4% of the beta diversity variation was explained by a Corsica vs Continent difference, 4% by the difference between Italy and France, 11% by the host species and 21% by the subgenus. In all, 41% of beta diversity variation was explained by these factors (Table 2). The strongest differences illustrated by the NMDS were between *A. cordata* communities in Italy and Corsica, and between *A. alnobetula* subspecies and the two other alder species (Fig. 4c). Subsequent tests confirmed that *A. alnobetula* hosted less variable communities than other hosts (ANOVA on betadisper test, $P = 0.047$ and 0.008699 for EM fungi and *Frankia*, respectively) and that communities were as variable in Corsica as on the continent (ANOVA on betadisper test on region level 1, $P = 0.897$ and 0.8518 for EM and *Frankia*, respectively).

Climatic variables had different effects on EM fungi and *Frankia* communities, the NMDS structure being significantly correlated with twice as many variables for *Frankia* than for EM (Table S6; Fig. 4b,d). The effects of longitude and elevation were significant for EM and *Frankia*, and latitude for EM only (Table S6). For *Frankia*, bioclimatic and geographic variables were correlated with the host subgenus and species factors (Fig. 4d). Soil variables were not included, due to the lack of data available for several sites (Table S2).

Species distribution and detection of endemism and specificity

For fungi, 37 out of 110 species were restricted to Corsica – of which 12 were observed only once – and six were unique to Italy. For *A. cordata*, 33 of 44 species were exclusive to Corsica (Fig. 5). Two of these (*Clavulina* sp. 1 and *Russula* sp. 7) can be considered as potential endemics of Corsica as they have never been found before. For *A. alnobetula* subsp. *suaveolens*, six out of 17 species were restricted to Corsica (Fig. 5). Two hypogeous species, already described in Corsica, *Alpova corsicus* and *Melanogaster rivularis,* were true endemics. For *A. glutinosa*, only seven out of 24 species were restricted to Corsica in our dataset but they were mostly unnamed and therefore their endemism remains to be proved. For *Frankia*, four species were restricted to continental France, three to Italy and 10 were endemic to Corsica, of which seven were observed only once (Table S4). *A. glutinosa* hosted all the endemic *Frankia* species and shared three species with *A. cordata*, while *A. alnobetula* subsp. *suaveolens* did not host any endemic species (Fig. 5). Hierarchical clustering showed that the distribution of EM fungi and *Frankia* species was explained first by host and then by region.

The specialization class of each species is reported in Tables S3 and S4 for EM fungi and *Frankia*, respectively. Over the whole dataset, 20% of EM species were quoted as undetermined and 35% as generalists. All *Frankia* species were strictly associated with alders, and not considered as generalists – a term applied here only to fungi. Eight per cent of *Frankia* and 15% of EM species were alder-specialists, 25% of *Frankia* and 17% of EM species were subgenus *Alnus*-specialists, 42% of *Frankia* and 2% of EM species were *A. glutinosa*-specialists, 16% of *Frankia* and 4% of EM species were *A. cordata*-specialists and 8% of *Frankia* and 7% of EM species were *A. alnobetula*-specialists. Over the sites studied, the positions of specificity class factors were similar for both symbionts except for alder-specialists (Fig. 4e). Communities associated with *A. glutinosa* in Corsica did not appear more specialized than those on the Continent. By contrast, both *Frankia* and EM specificity was reduced in Corsica compared to Italy (Fig. 4e). The higher proportion of symbionts specific to the *Alnus* subgenus was significantly correlated with several bioclimatic variables related to warmer and dryer conditions. None of the soil variables tested was significant (Fig. 4f; Table S6).

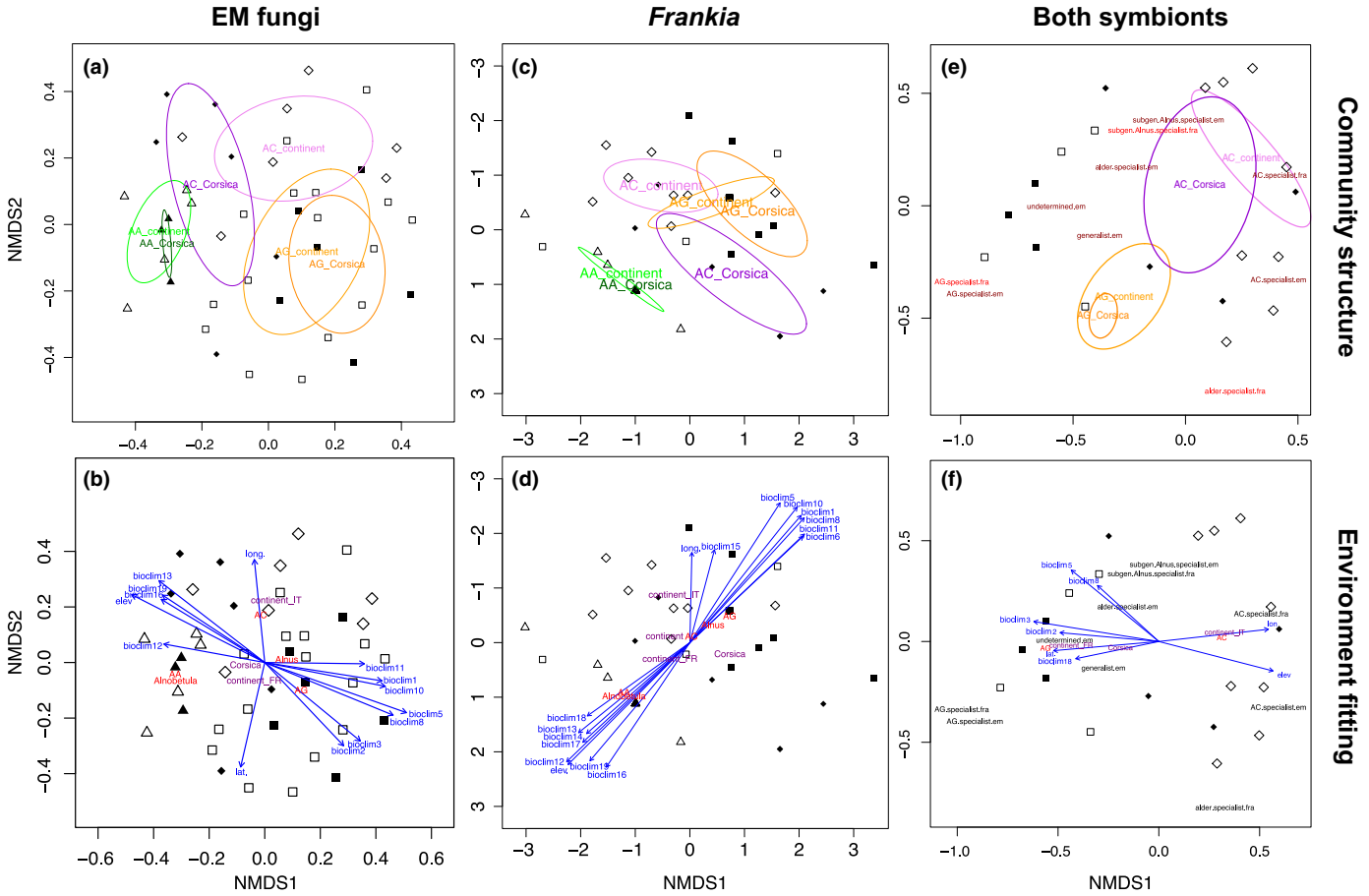

Fig. 4 Community structures and environment fitting for ectomycorrhizal (EM) fungi and *Frankia*. Community structure of (a) EM fungi, (c) *Frankia* and (e) both symbionts. Fitting of environmental variables and factors for (b) EM fungi, (d) *Frankia* and (f) both symbionts. (a, c, e) The effect of alder host species (*Alnus alnobetula*, AA; *A. cordata,* AC; *A. glutinosa*, AG) and regions (Corsica vs Continent) on site ordination is illustrated by nonmetric multidimensional scaling (NMDS). For (a) EM fungi and (c) *Frankia*, two (nos. 43 and 59) and four (nos. 14, 15, 24 and 47) outlying populations were removed, respectively; for (e) both symbionts, only alder populations in common were included except three *Alnus alnobetula* populations that were discarded. (b, d, f) The correlation between NMDS, host and regional factors as well as geographic and climatic variables was tested through a permutation analysis (envfit function). (f) Four soil variables were also tested. Only significant factors ($P > 0.05$) are represented (see Table S6 for details).

Distribution of short-distance dispersal symbionts

Spore-positive (Sp+) *Frankia* were identified in all regions and host species. In Corsica and on the Continent, *Frankia* nodules were 100% Sp+ vs 69.2% for *A. alnobetula*, 12.8% Sp+ vs 45.7% for *A. glutinosa*, and 86.1% Sp+ vs 35.3% for *A.* cordata, respectively. These proportions were significantly different between Corsica and the Continent (two-sample test with continuity correction, all $P < 0.01$). Species richness was partly explained by the Sp+ frequency (linear model, $P < 0.1$; adjusted $R^2 = 25$–50% according to the regression method) and these two parameters were negatively correlated (Spearman rank-order correlation, $P < 0.01$; $\rho = -0.90$).

Seven genera of hypogeous fungi were detected: *Alpova, Hydnobolites*, *Melanogaster*, *Octaviania*, *Pachyphlodes* and *Tuber* spp. The frequency of the hypogeous fungi group (based on the number of sequences) was higher in Corsica than on the Continent (ANOVA, $P = 0.03$). However, the predominance of hypogeous fungi in Corsica, as compared to Italy, was significant for *A. cordata* (Mann–Whitney test on hypogeous frequency, $P = 0.02$, Fig. S3), but not for the two other alder species (Mann–Whitney tests on hypogeous frequency, $P = 0.62$ for both). Of interest, *Cenococcum geophilum*, a common and widespread ascomycete that does not produce fruiting bodies, was found only twice in Corsica.

Discussion

Based on classical hypotheses on islands and ice-age refugia, we expected to detect higher alpha and gamma diversity of symbiont communities on Corsican alders, and following recent studies on alders, we also expected more endemic species of *Frankia*. Interestingly, Corsica harbours a high level of diversity in some cases, but the patterns of distribution were far more complex than assumed. This was because geographic isolation had a different effect on each symbiont

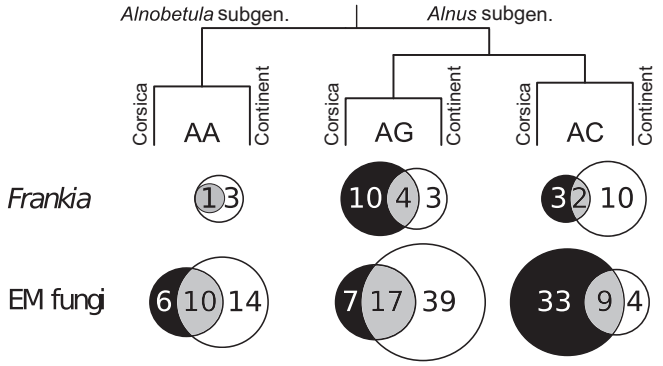

Fig. 5 Distribution of ectomycorrhizal (EM) fungi and *Frankia* species among regions and hosts. The clustering was based on community similarity (Bray–Curtis distance). The Venn diagrams show, for each symbiont and each host, the number of species in Corsica and on the Continent, as well as the number of species shared between the two regions. Hosts, *Alnus alnobetula* (AA), *A. cordata* (AC), *A. glutinosa* (AG).

community, which in turn also depended on the host species. This suggests that the theory of island biogeography may not apply in every case.

Diversity of symbiont communities

Corsican *Frankia* communities were more diverse on *A. glutinosa*, as previously reported on *A. glutinosa* subsp. *barbata* from the Colchis region, another glacial and Tertiary refugium for alders (Roy *et al.*, 2017). Despite a similar sampling effort in Corsica, the number of *Frankia* species on *A. cordata* was three times lower than on *A. glutinosa.* This difference can be partly explained by the sporulation phenotype. Most strains isolated from *A. glutinosa* in Corsica were Sp− while most strains from *A. cordata* were Sp+, confirming that the percentage of Sp+ nodules on alder stands negatively correlates with *Frankia* diversity at a regional scale, as already shown in Europe and North America (Pozzi *et al.*, 2015). As Sp+ strains are highly host-dependent and produce a huge number of clonal offspring, they would invade the roots locally (Cotin-Galvan *et al.*, 2016) and could prevent hosts from associating with other *Frankia* strains, creating a bottleneck effect in *Frankia* diversity. Conversely, Corsican EM communities were richer on *A. cordata,* while it was not the case in a previous study (Roy *et al.*, 2013). The number of EM species on *A. cordata* was twice that on *A. glutinosa.* This difference was due to an increased recruitment of generalist EM fungi rather than a recruitment of unique alder specialist EM fungi by *A. cordata,* a situation which would be easily achieved in Corsica where the ecological range of *A. cordata* includes mixed forests. In summary, communities were distinct between regions, and shaped by their geographic position, confirming that the spatial structure of alder symbiont communities could be relatively strong at the regional scale, as previously demonstrated for EM (Peay *et al.*, 2007, 2010) and for both symbionts, especially in refugia (Roy *et al.*, 2017). However, regional differences were not necessarily explained by the recruitment of endemic symbionts.

EM and *Frankia* endemism

While 37 EM species were detected only in Corsica, only four can be considered to be true endemics after database queries: the two hypogeous fungi *Alpova corsicus* and *Melanogaster rivularis* (Moreau *et al.*, 2011), one unidentified *Russula* and one *Clavulina* species – '*Clavulina* sp. 1', specific to *A. cordata*. Interestingly, these last two species are epigeous (our unpublished observations for *Clavulina* sp. 1) and supposedly wind-dispersed, but they have not been found associated with *A. cordata* on the continent. They were not found in native stands in Calabria either, unlike the most common *A. cordata*-specialist, *Alnicola salabertii*, which is present wherever its host can be found (Moreau & Garcia, 2005). The remaining 33 EM species were generalist fungi previously recorded outside of Corsica, such as *Amanita rubescens* or *Russula delica*. These data confirm that alder stands in Corsica host no more than a few endemic EM species (Taudiere *et al.*, 2015). Similarly, a study on polypores in Corsica also failed to detect endemic taxa (Nemergut *et al.*, 2001). According to a review on EM fungi, isolation by distance was only detected for wind-dispersed populations separated by thousands of kilometres (Douhan *et al.*, 2011). All these observations suggest that geographic distance and time of separation between Corsica and the European continent were not sufficient to induce speciation for EM fungi, with the exception of a few hypogeous species which might be paleoendemics rather than allospeciated (Moreau *et al.*, 2011).

As *Frankia* may be more dispersal-limited than EM fungi, we expected unique *Frankia* species and richer communities in Corsica. Indeed, 50% of the recovered *Frankia* species have never been recorded on the continent or in previous samples (Pozzi *et al.*, 2015; Roy *et al.*, 2017) and were therefore considered to be endemic to the island. Surprisingly, the more widespread was the alder species, the more endemic *Frankia* it hosted in Corsica: *A. glutinosa* hosted more endemic species than *A. cordata*, while the strictly Corsican *A. alnobetula* subsp. *suaveolens* hosted no endemic species (Fig. 5). However, although all *A. alnobetula* strains constituted a unique species (clade 1a) in the phylogeny (Fig. 3), Corsican strains clustered together (clade 1a AAS), suggesting the existence of an endemic lineage at a taxonomic rank lower than species. The divergence between Corsican and continental *A. alnobetula* populations only 1.1 Ma (Rochet *et al.*, 2011) was probably not sufficient to allow speciation of *Frankia* and detection of endemic species. At the opposite end of the spectrum, the oldest divergence in *Frankia* phylogeny corresponded to clade 8. This group is entirely associated with *A. cordata*, a species that diverged from other alders 22.9 Ma (Rochet *et al.*, 2011). The position of Italian strains in this clade suggests a later colonization from Corsica, possibly across the terrestrial bridge connecting Corsica to Italy during the Messinian crisis 5 Ma (Clauzon *et al.*, 1996). This past exchange between Corsica and Italy may explain the current reduced endemism found on *A. cordata* in Corsica. For *A. glutinosa*, the presence of numerous endemic species of *Frankia* found in Corsica confirms the pattern recently described in another Tertiary and glacial refugium, the Colchis region (Roy *et al.*, 2017). However, for

Corsica, *A. cordata* and *A. glutinosa* occur sympatrically at mid-altitudes, where they could hybridize (Lhote, 1985; King & Ferris, 2000; Gryta *et al.*, 2017), explaining why endemic strains can be shared between the two hosts.

Patterns of specialization

In Corsica, the co-occurrence of three alder species and the growth of *A. cordata* in mixed woods represent a rare situation for EM fungi, as alders generally contribute little to networks (Kennedy *et al.*, 2015; Taudiere *et al.*, 2015). We indeed detected shared symbionts in Corsica, but also on the continent and not necessarily at the scale of alder stands. Interestingly, we detected *Frankia* species shared between *A. glutinosa* and *A. cordata* both in Corsica and on the continent (for instance, OTU 20). This pattern of *Frankia* co-occurrence was relatively rare, as 66% of *Frankia* species are only associated with a single alder species in our dataset. This was considerably more than the 13% for EM fungi. Only 8% of *Frankia* species were found to be associated with the three studied alder species, compared to 17% in EM fungi. Thus, *Frankia* species appeared to be more host-specific than EM species. Among EM fungi, 10 species belonging to the genera *Alnicola*, *Lactarius* and *Tomentella* were shared between alder species both on the continent and in Corsica. Only two EM species were shared only in Corsica, *Cenococcum geophilum* – a generalist – and *Pachyphlodes* sp. Therefore, the distribution of alders in Corsica might not promote recent host shifts or sharing, in line with observations on the continent. The analysis of EM specificity confirmed that *A. cordata* hosted 'more generalist' communities in Corsica, compared with pure *A. cordata* stands like those in Italy. Although the sequencing rate was lower on Italian populations than in Corsica, the species accumulation curve of *A. cordata* in Italy was saturated, suggesting that sampling bias may not explain the difference in specificity. We did not detect a correlation between community specialization and any soil variable tested in our study, consistent with the literature (Põlme *et al.*, 2013; Rochet *et al.*, 2011). Our results suggest rather that a warmer and drier climate correlates with less specialized, more generalist symbiont communities. Numerous ascomycetes were isolated from *A. subcordata* (C.A.Mey) Regel in Iran (Põlme *et al.*, 2013) and *A. glutinosa* subsp. *barbata* (C.A.Mey) Yalt. in Georgia (Roy *et al.*, 2017). These two alder species are Tertiary relics of the Hyrcanian forest and the Colchis region, and colonize relatively dry and warm habitats, like *A. cordata* in Corsica. Interestingly, Corsican *A. cordata* hosted more ascomycetes and hypogeous taxa than in Italian populations or other hosts. Hypogeous taxa are also supposedly well adapted to drought (Richard *et al.*, 2011; Herzog *et al.*, 2013) and are abundant in Mediterranean or dry habitats (for a review see Zambonelli *et al.*, 2014). Sequences of *Tuber* sp. were detected on these three alder species, and also on *A. glutinosa* introduced to New Zealand (Bogar & Kennedy, 2013; Bogar *et al.*, 2015). None of these sequences is identical and their detection on a few sites suggests local recruitment rather than true specialization on alders.

Conclusion

Study of the *Frankia*–EM–alder symbiosis in Corsica is an interesting model, permitting finer analysis at the community level. We detected a high level of endemism for *Frankia* but not for EM fungi. Our results also revealed that symbionts which are host-specialists or poor dispersers have distinct biogeographic patterns. Yet, it is difficult to fully understand the biogeography of this tripartite symbiosis at a regional scale, perhaps because several dispersal events contributed to the colonization of Corsica and because gene flow with populations on the continent remains important at least for epigeous fungi. While Corsica is well known as a hotspot of plant diversity, comparison with Italy clearly showed that other alder stands can also host diverse *Frankia.* Finally, specificity patterns of both symbionts followed a common trend and did not increase particularly in Corsica, suggesting that specificity rarely varies at these geographic and temporal scales. Only *A. cordata* clearly associated with more generalist EM fungi and more host-dependent Sp+ *Frankia*, which raises new questions about the ecology of this peculiar host.

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Author contributions

All authors contributed to this work. A.C.P., M.R., P-A.M., M.G. and M.P.F. designed the research and collected new samples in Corsica. A.C.P., M.N., G.S. and S.M. performed the laboratory work. A.C.P., M.R. and M.N. analyzed the data. A.C.P., M.R., P-A.M. and M.P.F. interpreted the data and wrote the manuscript. A.C.P. and M.R. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Histogram of pairwise phylogenetic distances for *Frankia* species delineation.

Fig. S2 Ectomycorrhizal (EM) fungi and *Frankia* species accumulation curves.

Fig. S3 Frequency of ectomycorrhizal (EM) fungi reproducing below ground (hypogeous taxa, *Tylospora*, *Amphinema*, *Piloderma* and *Cenococcum geophilum*) and Ascomycota sequences.

Table S1 Sampled population information and new sites sampled in this study

Table S2 Soil variables for the most recent sampled sites

Table S3 Community matrix of ectomycorrhizal (EM) fungi species and GenBank accession numbers

Table S4 Community matrix of *Frankia* species

Table S5 Community matrix of ectomycorrhizal (EM) fungi and *Frankia* specialization classes for the commonly sampled populations

Table S6 Synthesis on environmental variables tested in NMDS

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